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Sialic acid is an essential moiety of mucin as a hydroxyl radical scavenger

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Abstract In this work, we examined the antioxidant role of mucin, a typical sialic acid containing high-molecular weight glycoprotein. The function of mucin as a hydroxyl radical (·OH) scavenger was characterized using bovine submaxillary gland mucin (BSM). Non-treated BSM effectively protected DNA from the attack of ·OH; however, desialylated BSM lost this potential. Moreover, we estimated the scavenging effects of BSM against ·OH generated by UV irradiation of hydrogen peroxide using ESR analysis. Our results indicate that BSM has ·OH scavenging ability the and sialic acid in mucin is an essential moiety to scavenge ·OH.

Keywords: Oxidative stress; Mucin; Reactive oxygen species; Sialic acid; Hydroxyl radical

1. Introduction

Hydroxyl radical (·OH) is known as one of the most highly reactive and harmful oxygen derived free radicals in a living organism [1]. This radical reacts with various cellular components including lipid, protein and DNA to oxidatively modify or decompose them [2,3]. It has been proposed that mucus layers lining the respiratory tract and gastrointestinal system may be physiologically important free radical scavenger [4]. The surface epithelium of both the respiratory and gastrointestinal tract is covered by an extracellular and renewable layer of gelatinous mucus. This mucus contains mucin, uric acid, ascorbic acid and reduced glutathione (GSH). Mucins are large, abundant, complicated, filamentous glycoproteins that are present at the interface between many epithelia and their extracellular environments [5,6]. Salivary mucins, which are well known large glycoproteins act as a functional barrier capable of modulating the untoward effects of the oral environment [7,8]. Halliwell and co-workers showed that when pig gastric and respiratory tract glycoproteins were reacted with systems that generate ·OH there was an increase in thiobarbituric acid reactivity which is indicative of oxidative damage to the carbohydrates [9,10]. Interestingly, recent studies suggest that mucin synthesis is induced by oxidative stress [11,12]. However, there have been no intensive studies to elucidate the relation between mucin and ·OH or other reactive oxygen species (ROS) to date. Therefore, in order to characterize the antioxidant activity of mucin against ·OH, we examined the role of sialic acid on the terminal position in the glycoprotein using bovine submaxillary mucin (BSM) under physiological conditions.

2. Materials and methods

2.1. Chemicals

BSM and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1,2-Diamino-4,5-methylenedioxybenzene (DMB), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 4-hydroxy-2,2,6,6-tetramethyl-piperidine-*N*-oxide (TEMPOL) and hydrogen peroxide (atomic absorption grade) were obtained from Wako Pure Chemical Co. (Osaka, Japan). Sialidase (*Arthrobactor ureafaciens*) was from Nakarai tesque (Kyoto, Japan). All other reagents were of the highest grade commercially available.

2.2. DNA strand cleavage assay

The DNA-nicking assay was performed according to the method of Kukielka [13,14] with minor modification using pBluescipt II SK$^-$ DNA. Hydroxyl radicals were generated by incubating the following reagent at the indicated final concentrations in 0.5 ml of PBS (pH 7.4) at 37 °C for 20 min: 50 μM H_2O_2, 5 μM $FeCl_3$, 25 μM EDTA, 10 μM ascorbic acid and 0.5 μg of DNA. The iron salt was premixed with EDTA before addition to the reaction mixture and the reaction was started by the addition of ascorbic acid.

2.3. Electron spin resonance measurement

A reaction mixture containing 10 mM DMPO, 0.3% H_2O_2 and test samples in 250 μl was prepared in a 1.5 ml test tube. The mixture was transferred to a cell and illuminated at 365 nm using a PAN UV lamp. After 20 s of UV-irradiation, the DMPO-OH spin adduct was measured with a JEOL 200ESR spectrometer (X-Band Microwave Unit, JEOL, Japan). ESR measurement conditions were the following: microwave power 8 mW, microwave frequency 9.45 GHz, modulation amplitude 0.08 mT, time constant 0.03 s, sweep time 1 min, sweep width 5 mT and center field 335.95 mT. The signal intensity of DMPO-OH was normalized against Mn^{2+} signal, which was used as an internal standard. The component signals observed in these spectra were identified and quantified as reported [15,16]. The double integrals

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Abbreviations: BSM, bovine submaxillary gland mucin; DMB, 1,2-diamino-4,5-methylenedioxybenzene; ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; NANA, *N*-acetyl neuraminic acid; NGNA, *N*-glycolyl neuraminic acid; ROS, reactive oxygen species; TEMPOL, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-*N*-oxide

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of DMPO-OH experimental spectra were compared with those of a 1 μM 4-hydroxy-2,2,6,6-tetramethyl-piperidine-*N*-oxide (TEMPOL) sample measured under identical settings to estimate the concentration of spin adduct.

2.4. Preparation of desialylated BSM

Desialylated BSM was prepared by treatment of intact BSM with sialidase (60 mU/ml) in 50 mM acetate buffer (pH 5.4). After 90 min incubation at 37 °C the solution was dialyzed in PBS to remove the hydrolyzed free NANA and NAGA. A control of BSM was treated in 50 mM acetate buffer without sialidase and the solution was dialyzed in PBS. After determination of core protein concentration of BSM in the dialyzed solutions, to confirm the complete desialylation of BSM, the NANA and NGNA contents were determined by the prelabeling HPLC assay using DMB [17] with acid hydrolysis of all sialic acid after saponification of BSM by treatment with 1.0 M KOH [18].

2.5. Determination of sialic acid in mucin following exposure of ·OH

BSM was exposed to hydroxyl radicals generated by incubation the following reagents at the indicated final concentrations in 0.5 ml of PBS (pH7.4) at 37 °C for 24 h: (1) 1 mM H_2O_2. (2) 20 μM $FeCl_3$, 100 μM EDTA and 50 μM ascorbic acid. (3) 1 mM H_2O_2, 20 μM $FeCl_3$, 100 μM EDTA and 50 μM ascorbic acid. Residual sialic acid concentrations were determined in all reaction mixtures and filtrates by the HPLC method described above.

2.6. Detection of free sialic acid released from BSM by liquid chromatography–mass spectrometry (LC–MS)

After exposure of BSM to the ·OH generation system (1 mM H_2O_2, 20 μM $FeCl_3$, 100 μM EDTA and 50 μM ascorbic acid) for 24 h, the reaction mixture was ultrafiltered. A portion of the filtrate was applied to LC–MS to detect free NANA and NGNA according to the method of Valianpour et al. [19] with minor modification. The HPLC system consisted of a Shimazu LC/MS (LCMS-2010A, Shimazu, Kyoto, Japan). The mass spectrometer was used in the negative electrospray ionization (ESI) mode. Sample (10 μl) was loaded on an Inertsil aminopropyl column (2.1 × 50 mm; 5 μm particle size; GL Sciences Inc. Tokyo, Japan).

2.7. Other method

Protein concentrations were determined by the method of Lowry [20] using bovine serum albumin as a standard.

2.8. Statistics

Each experiment was performed at least four times and the results are expressed as means ± S.D. Data were analyzed by an analysis of variance (ANOVA). Post-hoc comparisons of means between groups were performed using Bonferroni's correction with a significance level of 0.05.

3. Results

3.1. Protection of DNA from ·OH damage

As shown in Fig. 1, the effect of BSM on ·OH scavenging showed dose dependent behavior. In addition, free NANA and NGNA exerted a slight scavenging effect, however the effect was weak compared to BSM containing the same concentration of sialic acid (Fig. 1A). The DNA protection ability of sialic acids in mucin was equal to or higher than that of GSH and mannitol at same concentration (Fig. 1B).

When 2.67 mg/ml BSM containing 1 mM sialic acid was treated in 50 mM acetate buffer with or without sialidase, the concentration of residual sialic acid in BSM obtained after dialysis was 19.78 μM (desialylated BSM) or 820 μM (Control). Thus, we used desialylated BSM in which over 97.5% of sialic acids was removed compared to control BSM. As shown in Fig. 2, BSM treated in acetate buffer has a scavenging potential, while these effects were not observed with desialylated BSM.

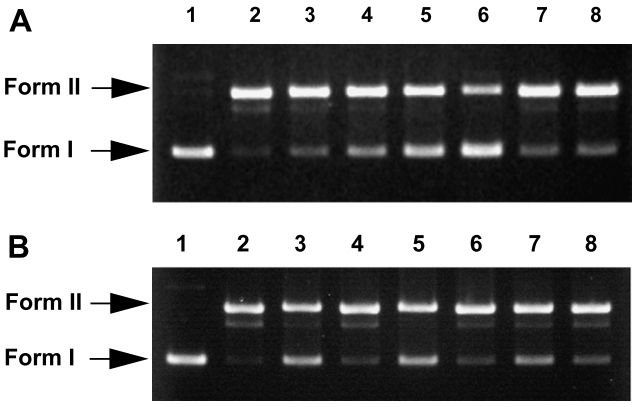

Fig. 1. Dose–response and efficacy of BSM on DNA damage induced by ·OH exposure. Electrophoresis of plasmid using agarose gel (1.0%) performed following exposure to ·OH generated by Fenton reaction. Experiments were carried out for 20 min at 37 °C using Fenton reaction mixture (final concentrations in 50 μl of PBS: 50 μM H_2O_2, 5 μM $FeCl_3$, 25 μM EDTA, 10 μM ascorbic acid) A: Lane 1, plasmid; lane 2, Fenton reaction mixture plus plasmid; lane 3, Fenton reaction mixture plus plasmid and 0.14 mg/ml BSM containing 50 μM sialic acid; lane 4, Fenton reaction mixture plus plasmid and 0.27 mg/ml BSM containing 100 μM sialic acid; lane 5, Fenton reaction mixture plus plasmid and 0.54 μg/ml BSM containing 200 μM sialic acid; lane 6, Fenton reaction mixture plus plasmid and 1.08 mg/ml BSM containing 400 μM sialic acid. lane 7, Fenton reaction mixture plus plasmid and 200 μM NANA; lane 8, Fenton reaction mixture plus plasmid and 200 μM NGNA. B: Lane 1, plasmid; lane 2, Fenton reaction mixture plus plasmid; lane 3, Fenton reaction mixture plus plasmid and 1 mM GSH; lane 4, Fenton reaction mixture plus plasmid and 100 μM GSH; lane 5, Fenton reaction mixture plus plasmid and 1 mM mannitol; lane 6, Fenton reaction mixture plus plasmid and 100 μM mannitol; lane 7, Fenton reaction mixture plus plasmid and 0.54 mg/ml BSM containing 200 μM sialic acid; lane 8, Fenton reaction mixture plus plasmid and 0.27 mg/ml BSM containing 100 μM sialic acid.

3.2. Scavenging activity against ·OH generated by UV irradiation of hydrogen peroxide

To detail the mechanism of ·OH scavenging ability by BSM, we estimated the effect on a transient metal independent ·OH generating system. In this experiment, we used completely desialylated BSM and the corresponding control BSM treated in acetate buffer without sialidase. Four characteristic signal lines were observed 20 s after UV irradiation of H_2O_2 with DMPO (Fig. 3A). The effects of BSM and desialylated BSM on DMPO-OH signal intensity were estimated in triplicate at the concentration of 0.16 mg/ml (corresponding to 60 μM sialic acid in control BSM). As shown in Fig. 3B, BSM significantly inhibited DMPO-OH spin adduct formation, while a significant effect was not observed with desialylated BSM.

3.3. Decrease of sialic acid in mucin by exposure to ·OH

Fig. 4 shows the total sialic acid contents (NANA plus NGNA) in the total reaction mixture after incubation with various substrates for ·OH generating reactions. Total sialic acid contents in BSM were significantly decreased following exposure to ·OH generated by the Fenton reaction, while significant decreases in sialic acid were not observed with iron (III) – ascorbic acid or hydrogen peroxide in the reaction mix-

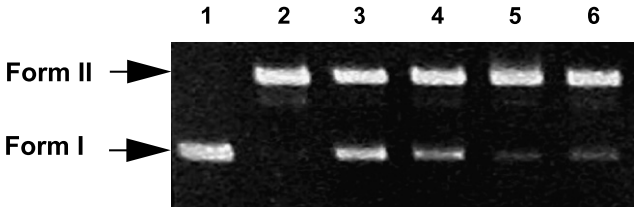

Fig. 2. Protection of hydroxyl radical-induced DNA damage by BSM. Electrophoresis of plasmid using agarose gel (1.0%) performed following exposure to ·OH generated by Fenton reaction. Experiments were carried out for 20 min at 37 °C using Fenton reaction mixture (final concentrations in 50 μl of PBS: 50 μM H_2O_2, 5 μM $FeCl_3$, 25 μM EDTA, 10 μM ascorbic acid). BSM was treated in acetate buffer with (desialylated BSM) or without (control) sialidase. Lane 1, plasmid; lane 2, Fenton reaction mixture plus plasmid; lane 3, Fenton reaction mixture plus plasmid and 0.54 mg/ml BSM containing 200 μM sialic acid; lane 4, Fenton reaction mixture plus plasmid and 0.27 mg/ml BSM containing 100 μM sialic acid; lane 5, Fenton reaction mixture plus plasmid and desialylated BSM (corresponding to intact BSM containing 200 μM sialic acid); lane 6, Fenton reaction mixture plus plasmid and desialylated BSM (corresponding to intact BSM containing 100 μM sialic acid).

ture. Over 20% of the sialic acid disappeared by the complete Fenton reaction system generating ·OH.

3.4. Determination of free sialic acid following exposure of BSM to ·OH

To investigate the liberation of free sialic acid by exposure of BSM to ·OH, the compounds generated by the reaction between BSM and ·OH were analyzed by HPLC with on-line MS. Fig. 5 shows the chromatograms when the standard free sialic acids and ultrafiltrates obtained after the reaction of BSM and ·OH generation system were applied to LC/MS. ESI produces negatively single-charged [M−H] ions for NANA (*m*/*z* 308.1) and NGNA (*m*/*z* 324.1), which were selected as parent ions. As shown in Fig. 5, free NANA and NGNA were not detected in the ultrafiltered fraction containing low molecular weight components.

4. Discussion

Sialic acids are known to exist in animals and occupy the terminal position of many glycoproteins. An earlier study suggested that ·OH reacted with a wide range of sugars including mannitol, fructose, galactose and sialic acid [21]. Therefore, it seems that high contents of sugars in mucus secretions should give them a substantial capacity to scavenge hydroxyl radicals [4]. Grisham et al. demonstrated that hydroxyl radicals derived from the iron-catalyzed decomposition of hydrogen peroxide are responsible for the depolymerization of native mucin [22]. Moreover, Cooper et al. described that peroxide attack on glycoproteins occurs largely at the histidine residues, with simultaneous peptidolysis. They also suggested that the mechanism most probably involves the liberation of hydroxyl radical by the Cu^+/Cu^{2+} dependent oxidation–reduction cycle [23]. Alternatively, Iijima et al. demonstrated that free NANA consumed toxic hydrogen peroxide, but NANA located at the non-reducing end of glycoprotein does not react with hydrogen peroxide [24]. However, most sialic acid is abundant as the terminal sugar of sialoglycoprotein and sialoglycolipids in vivo. Further, a recent report indicated that the

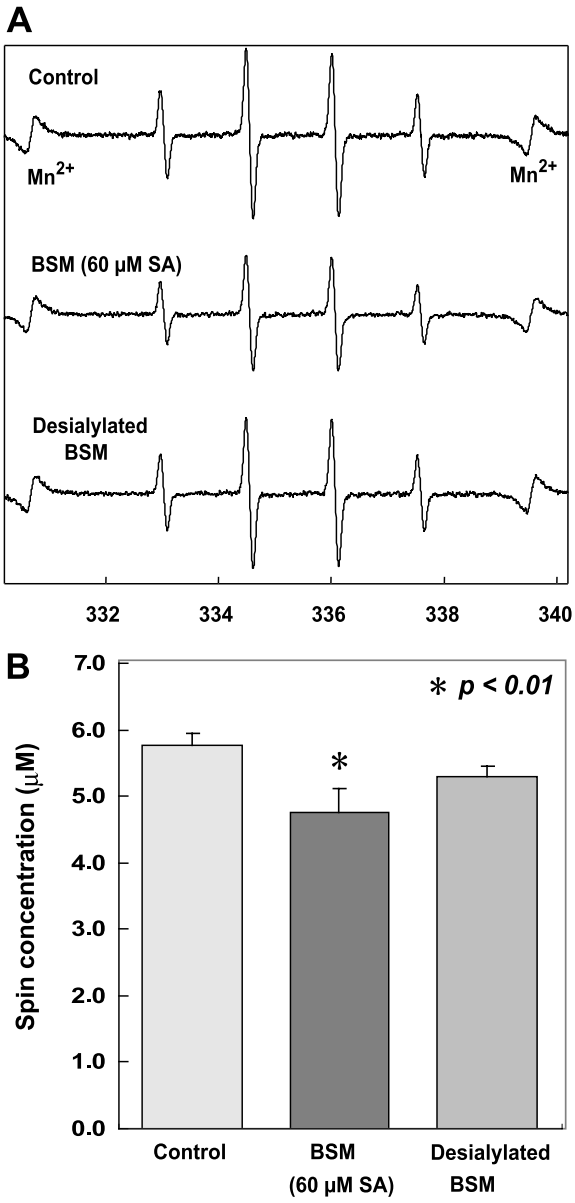

Fig. 3. Scavenging activity of BSM against ·OH generated by UV irradiation of hydrogen peroxide. BSM was treated in acetate buffer with (desialylated BSM) or without (BSM containing 60 μM sialic acid) sialidase. (A) ESR spectra observed on DMPO-OH formation with BSM (0.16 mg/ml) and desialylated BSM (0.16 mg/ml). Reaction mixture contained 10 mM DMPO, 0.3% H_2O_2 and test samples in 250 μl. Concentration of desialylated BSM was adjusted as core protein contents of intact BSM. (B) The effects of BSM on DMPO-OH signal intensity were estimated at the concentration of spin adduct, and data are presented as the means ± S.D. of triplicate determinations.

glycosidic linkage of sialic acid is a potential target for superoxide and other related ROS [25]. This study also proposed that ROS may regulate cell adhesion via the structural alteration of sialylated oligosaccharides on the cell surface. Clearly, it is important to unambiguously determine the moiety that is essentially required to exert ·OH scavenging action by mucin or glycoproteins under physiological conditions. Our aim was to measure the role of the terminal sialic acid of mucin for its ·OH scavenging ability. In the present study, we used

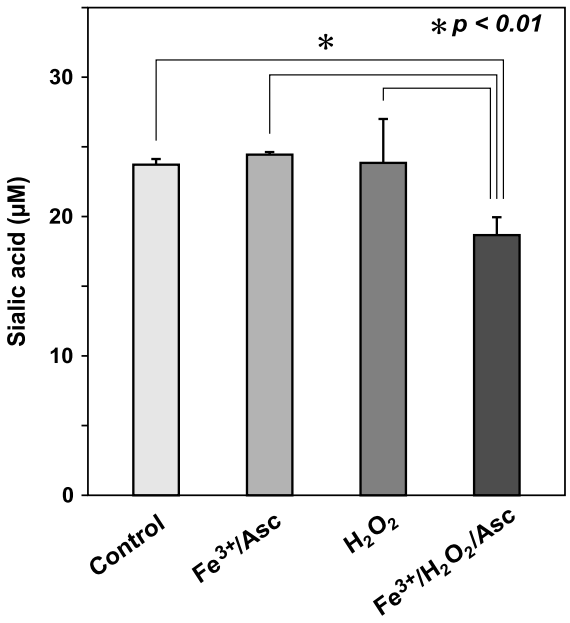

Fig. 4. Alteration of sialic acids in mucin treated with various components of the Fenton reaction. BSM (0.065 mg/ml) containing 25 μM sialic acid was treated with hydrogen peroxide (H_2O_2), 50 μM ascorbic acid, 50 μM Fe^{3+} and 250 μM EDTA (Fe + Asc), complete system (H_2O_2 + Fe^{3+} + Asc) and only vehicle (control). Total sialic acid (NANA plus NGNA) in the reaction mixture was determined by the prelabeling HPLC method described in Section 2.

sialidase to selectivity remove NANA and NGNA from glycoproteins under mild conditions. By comparing intact BSM and desialylated BSM in the assay to estimate ·OH scavenging ability, it was clearly shown that the sialic acids are critical components of the ability of mucin to act as a ·OH scavenger. We also determined that the core protein as well as its amino acid residues, have no direct participation in the scavenging ability under mild radical-generating systems that physiologically relevant in our experiments. Moreover, our results show that a sialic acid residue on the terminal position of polysaccharide chain has more specific reactivity than free sialic acid toward ·OH. Therefore, we think that the scavenging ability of a sialic acid rich glycoprotein like mucin should be recognized as distinct from the effect of monosaccharides, for example mannitol.

In this study, we especially focused mucin as a ·OH scavenger in various mucus layers that coat epithelial cell surfaces. Our results clearly show that the ·OH scavenging potential of BSM depends on the sialic acid residue and is specifically exerted on a metal-independent ·OH generating system. Furthermore, sialic acids in mucin specifically react with ·OH; however, the oxidative products are unclear. Eguchi et al. demonstrated that superoxide anion and the related ROS specifically cleaved and liberate the free sialic acid using 4-methylumbelliferyl-galactoside [25], while Tanaka et al. reported NANA in LDL was converted to unknown compounds during oxidation induced by Cu(II) and that the reaction was not a simple hydrolysis of the glycosyl bond of NANA [26]. Although our results show that a part of sialic acid in BSM disappeared after the attack of ·OH, liberation of free sialic acid was not observed in the ultrafiltrates by LC–MS. It was thought that liberated free sialic acid further reacted with excess H_2O_2 to form the oxidized products reported previously [24]. However, the compound produced by the reaction of free NANA and H_2O_2 was not found in our experiment using LC–

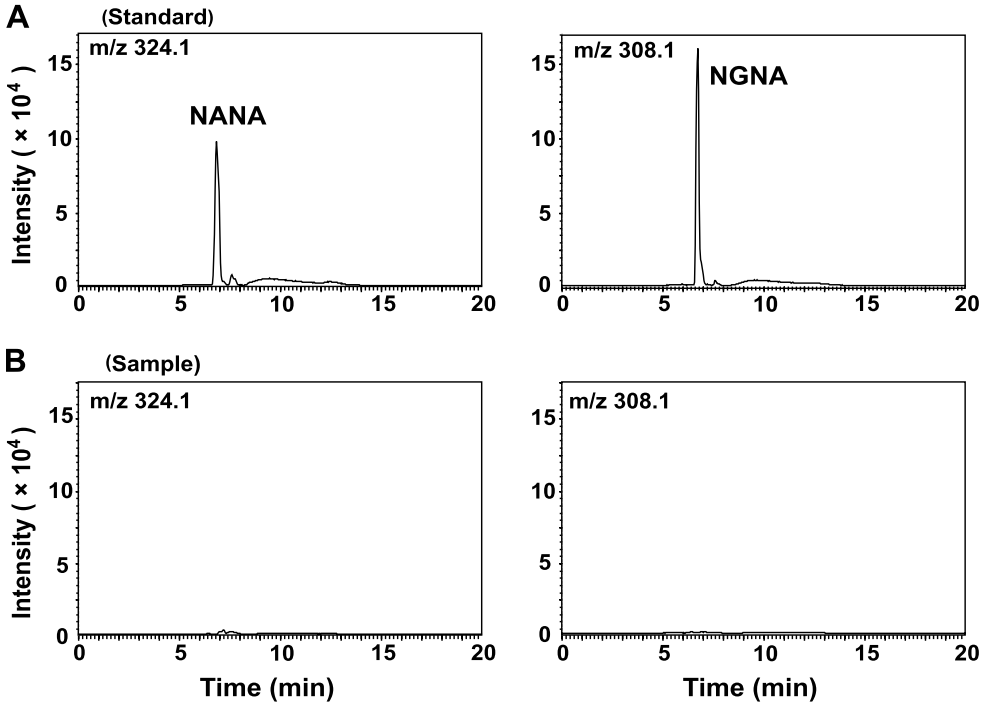

Fig. 5. Detection of NANA and NGNA following exposure of ·OH to BSM by LC–MS. A portion of the ultrafiltered reaction mixture obtained from BSM following exposure to the ·OH generating system for 24 h described in Fig. 4 was applied to LC/MS. Each sialic acid was monitored by ESI, which produced negatively single-charged [M−H] ions for NANA (*m*/*z* 308.1) and NGNA (*m*/*z* 324.1) as parent ions. A: standard solution (10 μM), B: sample solution.

MS analysis. Thus, it is unlikely the mechanism of the ·OH scavenging ability of mucin is based on the hydrolysis of glycosyl bond of sialic acid by ·OH. Further investigation using various glycoproteins containing sialic acid is required to detail the oxidative products formed by the reaction with ·OH.

In conclusion, it is strongly indicated that mucin acts as a sacrificial scavenger for ·OH and its protective function is exerted by the direct reaction with its sialic acids. It seems to be a reasonable biological response that ·OH generated by exogenous pollutant or xenobiotics from the environment are scavenged by the large amount of terminal sialic acid residues of mucin in the mucus layer covering the epithelial surfaces of the respiratory and gastrointestinal tracts.

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